

Modulation of Estrogen Receptor-mediated Transactivation by Orphan Receptor TR4 in MCF-7 Cells*

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The human testicular orphan receptor 4 (TR4) is a member of the nuclear receptor superfamily that shows a broad tissue distribution with higher expression in the nervous system and male reproductive tract. TR4 functions as a transcriptional modulator that controls various target genes via binding to the DNA hormone response elements. Here we report that instead of direct binding to hormone response elements for gene regulation, TR4 can also go through direct protein-protein interaction to repress estrogen receptor (ER)-mediated transactivation. Electrophoretic mobility shift and glutathione S-transferase pull-down assays clearly demonstrate that the direct interaction between TR4 and ER will inhibit the homodimerization of ER and interrupt/prevent ER binding to the estrogen response element. The consequence of these events may then result in the suppression of ER target genes, such as cyclin D1 and pS2 and inhibition of ER-mediated cell proliferation in the MCF-7 cells stably transfected with TR4. Together, our results showing that TR4 can suppress ER function via protein-protein interaction not only represent a unique cross-talk signaling pathway in the nuclear receptor superfamily, it may also provide us with a new strategy to modulate ER function in the breast cancer cells.

The orphan receptors belong to the nuclear receptor superfamily which mediates extracellular hormonal signals to transcriptional response. The roles of orphan receptors have been linked to development, homeostasis, and diseases (1–3). The human testicular receptor 4 (TR4)¹ was originally isolated from testes, prostate, and brain cDNA libraries by degenerative polymerase chain reaction cloning (4). While TR4 shares the structural features of nuclear receptors, no ligand has yet been identified, and it is therefore considered an orphan receptor. TR4 is highly expressed in the testes and prostate, as well as being widely expressed in the brain, particularly in the granule cells of the hippocampus and cerebellum (4).

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¹ The abbreviations used are: TR4, human testicular orphan receptor 4; DR, direct repeat; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; PPAR, peroxisome proliferator activated receptor; AR, androgen receptor; ER, estrogen receptor; SHP, short heterodimer partner; ERE, estrogen response element; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; E2, 17 β -estradiol; Dox, doxycycline; PR, progesterone receptor; LBD, ligand binding domain; EMSA, electrophoretic mobility shift assay.

TR4 directly regulates transcription through binding to a direct repeat (DR) of an AGGTCA core element separated by a variable number of nucleotides. TR4 functions as a transcriptional activator when bound to the DR separated by four nucleotides (a DR-4 element) (5). However, TR4 functions as a transcriptional repressor when bound to DR-1, DR-2, DR-3, or DR-5 type (6–8). The differential spacings between the core elements cause TR4 to adopt different conformations and alter the ability of TR4 to interact with coregulators (8). Consistent with its neuronal localization, TR4 also induces the transcription of a cytokine receptor, which is the ciliary neurotrophic factor receptor (9).

In addition to direct transcriptional regulation, TR4 can also modulate other nuclear receptors' transactivation. Previous studies have indicated that TR4 can compete for binding to the hormone response elements of retinoic acid receptor (RAR), retinoid X receptor (RXR) (6) and vitamin D receptor (VDR) (8) to suppress RAR/RXR- or VDR-mediated transcription. TR4 may also inhibit peroxisome proliferator activated receptor α (PPAR α)-induced transactivation by competitive binding to PPAR response elements and through competition for coactivators such as RIP140 (10). Recently, TR4 has been found to interact with the androgen receptor (AR). The AR-TR4 interaction could then result in the mutual suppression of AR- or TR4-mediated transcription (11). These studies suggest that TR4 could function as a master to modulate many signal pathways mediated by various nuclear receptors.

Estrogen receptors (ER) that play many essential roles for the growth in female reproductive tissues are encoded by two distinct genes, ER α and ER β (12). It has been demonstrated that ER α and ER β can form heterodimers (13), and ER α was able to directly bind to TR, RAR, RXR (14), short heterodimer partner (SHP) (15, 16), and ER β cx (17). ER α -TR and ER α -RXR heterocomplexes moderately enhance ER-mediated transcription in transient transfection experiments with CV-1 cells. In contrast, RAR repressed ER-mediated transactivation (14). The SHP inhibits ER transcriptional activity by preventing coactivator binding to ER (16), and ER β cx inhibits ER transactivation by preventing ER binding to DNA (17). Here we demonstrate that TR4 also inhibits ER transcriptional activity in lung cancer H1299 cells and in breast cancer MCF-7 cells. Further studies indicate that TR4 can suppress ER function via protein-protein interaction that results in the interruption of ER-ER homodimerization and in preventing ER binding to its estrogen response element (ERE).

MATERIALS AND METHODS

Plasmids—pCMV-TR4, pSG5-ER, pSG5-PR, pET-14b-TR4, pGEX-3X-TR4, pERE-CAT, and pMMTV-LUC were reported previously (9, 11, 18), and pBIG-2i and pCMV-mER β were gifts from Dr. Jay Reeder (University of Rochester, Rochester, NY) and Dr. Vincent Giguère (McGill University, Québec, Canada), respectively. For the GST fusion constructs, pGEX-3X-TR4-N was made by cloning the *SpeI/AatII* frag-

ment from pGEM-T EASY-TR4 into the *Sma*I site of pGEX-3X. pGEX-3X- Δ C-TR4 was made by removing *Hind*III/*Sac*I fragment of pGEX-3X-TR4. pGEX-3X-TR4-LBD was made by inserting the PCR-generated (amino acids 224–615) fragment of the human TR4 cDNA in the pGEX-3X vector. pGEX-2T-ER-LBD was made by inserting the ER cDNA fragment (amino acids 312–595) into pGEX-2T. The expression plasmid pCMV Δ C-TR4 was generated by deleting the *Pst*I fragment of pCMV-TR4. The pBIG-2i-TR4 was made by inserting the *Spe*I/*Not*I fragment of pGEM-T EASY-TR4 into *Spe*I/*Not*I site of pBIG-2i vector. All plasmids were verified by restriction enzyme analysis and DNA sequencing.

Cell Culture and Transfection—H1299 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (25 units/ml), streptomycin (25 μ g/ml), and 10% fetal calf serum (FBS). Transfections were performed using the calcium phosphate precipitation method, as described previously (11). Briefly, 3×10^5 cells were plated on 60-mm dishes for 24 h before transfection, and the medium was changed to DMEM with 10% charcoal/dextran-stripped FBS. H1299 cells were transfected with an ER expression plasmid (pSG5-ER or pCMV-mER β), ERE-chloramphenicol acetyltransferase (ERE-CAT), or pSG5-PR and MMTV-luciferase reporter plasmid, and a TR4 expression plasmid (pCMV-TR4). MCF-7 cells were only transfected with ERE-CAT reporter and TR4 expression plasmid. For all transfection experiments, pCMV β -gal (CAT assay) or pSV-40 RL (luciferase assay) was used as an internal control for transfection efficiency and the total amount of transfected DNA was adjusted to 12 μ g with pCMV. After 24-h transfection, the medium was changed again, and the cells were treated with 10 nM 17 β -estradiol (E2), progesterone, or vehicle as indicated. After another 24 h, the cells were harvested for CAT assay or Luciferase assay. The CAT activity was visualized by PhosphorImager (Molecular Dynamics) and quantitated by IMAGEQUANT software (Molecular Dynamics). The luciferase activity was determined by the Luminometer (Turner Designs).

Stable Transfection of MCF-7 Cells—The transfection plasmid was created by inserting the *Spe*I-*Not*I fragment of pGEM-T EASY-TR4 containing the full-length TR4, into the multiple cloning site of auto-regulated bi-directional tetracycline-responsive pBIG2i expression vector (19). MCF-7 cells were transfected with pBIG-2i-TR4 (MCF-7-TR4) or vector (MCF-7-pBIG) using the Superfect reagent (Qiagen), and the cells were maintained in DMEM, 10% FBS-selective medium containing 200 μ g/ml hygromycin (Invitrogen) for 2 weeks. Surviving cells were seeded onto 96-well cell culture plates. Cells were grown in selective media for an additional 2 weeks, and then individual colonies were picked and expanded in DMEM, 10% FBS.

Glutathione-S-Transferase (GST) Pull-down Assay—A GST pull-down assay was performed according to the methods described previously (11). Briefly, the GST fusion protein and GST control protein were expressed in an *Escherichia coli* strain BL21 (DE3) pLys bacterial culture and recovered on glutathione-Sepharose-4B beads. Equal amounts of GST fusion protein bound to glutathione-Sepharose-4B beads were incubated for 2 h at 4 $^{\circ}$ C with 5 μ l of *in vitro* translated [35 S]methionine-labeled protein in a total volume of 100 μ l of incubation buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mg/ml bovine serum albumin, and 10% glycerol) and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, leupeptin, and pepstatin). The beads were then washed three times with wash buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), boiled in 2 \times SDS sample buffer, loaded onto SDS-polyacrylamide gels, and visualized by autoradiography.

Northern Blotting Analysis—MCF-7-TR4 or MCF-7-pBIG cells were cultured in DMEM containing 10% charcoal/dextran-stripped FBS for 3 days, and then 3×10^6 cells were seeded on 100-mm dishes. After 24 h, the cells were treated with 2 μ g/ml doxycycline (Dox), a derivative of tetracycline, for 24 h and then treated for 48 h with 100 nM E2 or vehicle. Total RNA from cells was prepared by the ultracentrifugation method as described previously (6). A probe covering the N-terminal of TR4 was released by *Eco*RI and *Aat*II digestion and labeled with [32 P]dCTP using a random primer DNA labeling system (Amersham Biosciences). RNA samples (30 μ g) were electrophoresed and transferred onto a nylon membrane. The blot was hybridized with the human TR4 and β -actin sequentially and analyzed by autoradiography.

Western Blotting Analysis—MCF-7-TR4 or MCF-7-pBIG cells were cultured in DMEM containing 10% charcoal/dextran-stripped FBS for 3 days and then 1×10^6 cells were seeded on 100-mm dishes. After 24 h, the cells were treated with 2 μ g/ml Dox for 24 h and then treated with 100 nM E2 or vehicle. Cell lysates were collected after 12 h with or without E2 treatment by using RIPA buffer. The lysate protein amount

was quantitated by Bradford assay (Bio-Rad) with bovine serum albumin as a reference standard. One-hundred μ g of protein was loaded, and after electrophoresis on 10% SDS-PAGE was transferred onto Immobilon (Millipore, Bedford, MA). The membrane was blotted with anti-cyclin D1 rabbit polyclonal Ab (H-295) (Santa Cruz). Alkaline phosphatase-conjugated secondary Ab was used.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out as described previously (6). Briefly, 2.5 μ l of TNT-expressed ER with different amounts of TR4 was included in each reaction, and TNT lysate was used to make up equal amounts of lysate. The reaction was preincubated for 15 min at room temperature in 20 μ l of binding buffer (10 mM HEPES, pH 7.4, 50 mM KCl, 1 mM MgCl₂, 1 mM mercaptoethanol, 0.1 mM ZnCl₂, and 20% glycerol) containing 1 μ g of poly(dI-dC). [32 P]ATP end-labeled consensus ERE probes were added to the samples, incubated for 30 min at room temperature, and followed by another 30 min at 4 $^{\circ}$ C. For antibody supershift analyses, the reactions were incubated with 2 μ l of a monoclonal anti-ER antibody (C-314) (Santa Cruz) for 15 min at room temperature prior to the addition of probe. Protein-DNA complexes were resolved on a 5% native polyacrylamide gel and analyzed by autoradiography.

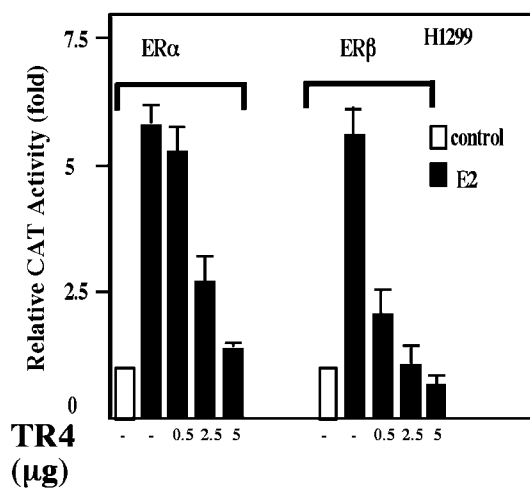
MCF-7 Cell Growth Assay—MCF-7-TR4 and MCF-7-pBIG cells, which were deprived of estrogen by culturing in phenol red-free DMEM medium supplemented with 10% charcoal-stripped FBS for 4 days, were plated at 10^4 cells/well into 24-well plate. After 24-h Dox treatment, 10 nM E2 was added to the cells. Cells were trypsinized at the specified time points and counted with a hemocytometer to determine cell density per sample.

RESULTS

TR4 Inhibits the Transactivation of ERs in Mammalian Cells—To determine the effect of TR4 on the modulation of ER-mediated transactivation, we used human lung cancer H1299 cells transfected with either ER α or ER β expression plasmid and cotransfected with increasing amounts of TR4 expression plasmid, and monitored the transactivation with an ERE-CAT reporter. As shown in Fig. 1A, with exogenous ERs, these cells became responsive to E2 by induction of ERE-CAT reporter activity in the presence of 10 nM E2. Cotransfection of wild-type TR4 with either ER α or ER β resulted in the suppression of the E2-induced CAT activity in a dose-dependent manner. As a control, we applied progesterone receptor (PR), which like ER also plays important roles in the mammary gland development (20), to demonstrate that TR4 has selective suppression with these two closely related nuclear receptors. As shown in Fig. 1A, while 10 nM progesterone can induce PR-mediated MMTV-LUC reporter activity, TR4 failed to suppress the transactivation of PR. The distinct difference in suppression of ER-mediated and PR-mediated transactivation suggests that these events are rather selective. This difference is also not an artifact due to a large amount of exogenously transfected TR4 plasmid, which may result in suppression of general gene transactivation. To further rule out the problem of potential artifacts from exogenously transfected ER, we also assayed TR4's ability to repress the MCF-7 endogenous ER-mediated transactivation. As shown in Fig. 1B, increasing the TR4 led to a gradual decrease in the endogenous ER-mediated CAT reporter activity. Together, data in Fig. 1 suggest TR4 is able to repress ER-, but not PR-mediated transactivation.

Interaction between TR4 and ER—To dissect the mechanism of how TR4 can repress ER transactivation, we used a GST pull-down assay. As shown in Fig. 2A, in the absence of E2, [35 S]methionine-labeled ER was able to interact with the GST-TR4 fusion protein but not with GST alone. This interaction is relatively specific for ER, as TR4 was unable to interact with RXR α , a common nuclear receptor that binds to many other nuclear receptors (1). Results from Fig. 2A are consistent with an earlier report showing TR4 fails to bind to RXR in mammalian two-hybrid assay (10). For the positive control, Fig. 2A also demonstrates that TR4 binds well to AR, a result consistent with a previous report (11). Fig. 2B further demonstrates that the LBD of ER interacts well with TR4 in the presence or absence of E2. In

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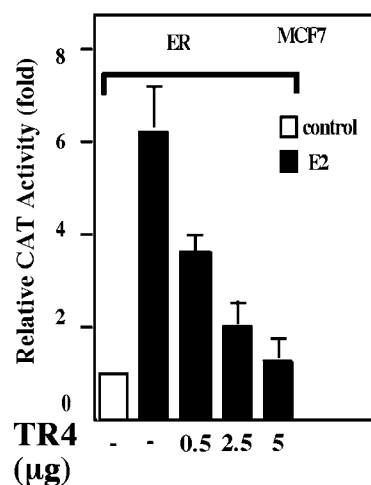


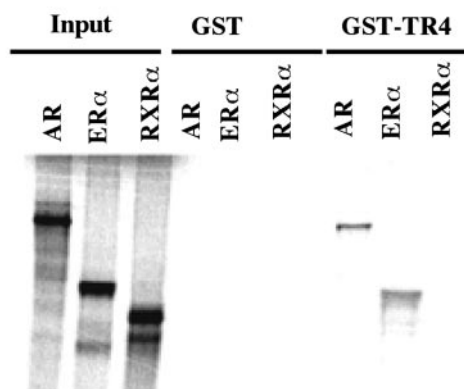
FIG. 1. **Selective suppression of ER transactivation by TR4.** A, H1299 cells were either cotransfected with ERE-CAT reporter plasmid and the expression plasmid for wild-type ER α or ER β or cotransfected with MMTV-LUC and PR plasmid, with increasing amounts of the TR4 expression vector in the absence or presence of 10 nM E2 (for ERE-CAT) or 10 nM progesterone (for MMTV-LUC). B, MCF-7 cells were cotransfected with ERE-CAT with increasing amounts of the TR4 expression vector. Cells were then treated with or without 10 nM E2 for ERE-CAT. The CMV- β gal (CAT assay) or SV-40 Renilla luciferase (luciferase assay) internal control plasmid was cotransfected to correct for transfection efficiency. The CAT activity fold was determined relative to activity in the absence of TR4 and E2. And the luciferase activity fold was calculated relative to activity in the absence of TR4 and progesterone. Bars represent the means \pm S.D. of three individual experiments.

summary, GST pull-down assays demonstrate that TR4 interacts with ER in the presence or absence of E2.

TR4-LBD Domain Is Essential for TR4 Suppression Effect on ER Transactivation—To dissect which domain within the TR4 can bind to ER, we generated GST-TR4 N-terminal, GST- Δ C-TR4, and GST-TR4-LBD fusion proteins (Fig. 3A) and tested their interaction with ER. As shown in Fig. 3B, only GST-TR4-LBD was able to interact with 35 S-labeled ER, but not GST-TR4 N-terminal and GST- Δ C-TR4. We then constructed an expression vector with a TR4 mutant lacking the C-terminal of LBD (pCMV Δ C-TR4) (Fig. 3A) to determine whether this mutant can still repress ER transactivation. As shown in Fig. 3C, while full-length TR4 could repress ER transactivation in a dose-dependent manner, the pCMV Δ C-TR4 showed no repression on the ER transactivation. Together, data from Fig. 3 demonstrate that the suppression effect of TR4 on ER relies on the physical interaction between two proteins, and the LBD within the TR4 C-terminal is an essential domain for TR4 to interact and repress ER transactivation.

Interruption of ER-ERE Binding and ER Homodimerization via LBD by TR4—To further dissect the molecular mechanism of how TR4 can repress ER target gene expression, we applied EMSA to investigate whether the inhibitory effect of TR4 is exerted at the level of ER-ERE binding. Fig. 4A, using *in vitro* expressed TR4 protein from rabbit reticulocyte lysate TNT system in gel-shift assay, demonstrates that the ER- 32 P-labeled ERE binding band (arrow) could be reduced by adding increasing amounts of TR4. TR4 could also reduce the amount of the ER-ERE supershifted complex in the presence of an ER α antibody (arrowhead). This finding clearly demonstrates that direct interaction between TR4 and ER will lead to interruption or prevention of the ER binding to ERE. To further test other possible mechanisms, we also assayed the influence of TR4 on ER binding with its coregulators or itself (homodimerization). Results in Fig. 4B demonstrate that TR4 can also inhibit ER-ER homodimerization. In contrast, TR4 shows little influence on the binding between ER and its coregulator, RIP140 (21) (data not shown).

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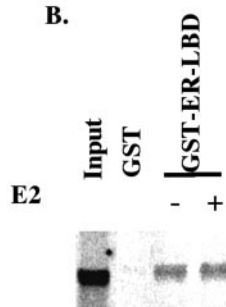


FIG. 2. **Interaction between TR4 and ER.** A, *in vitro* interaction of TR4 with ER, 35 S-labeled AR, ER, and RXR α were incubated with GST-TR4 or GST-bound glutathione-Sepharose beads in a pull-down assay. B, 35 S-labeled TR4 was incubated with GST-ER-LBD or GST-bound glutathione-Sepharose beads in the absence or presence of 1 μ M E2. The input represents 20% of the amount of labeled protein used in the pull-down assay.

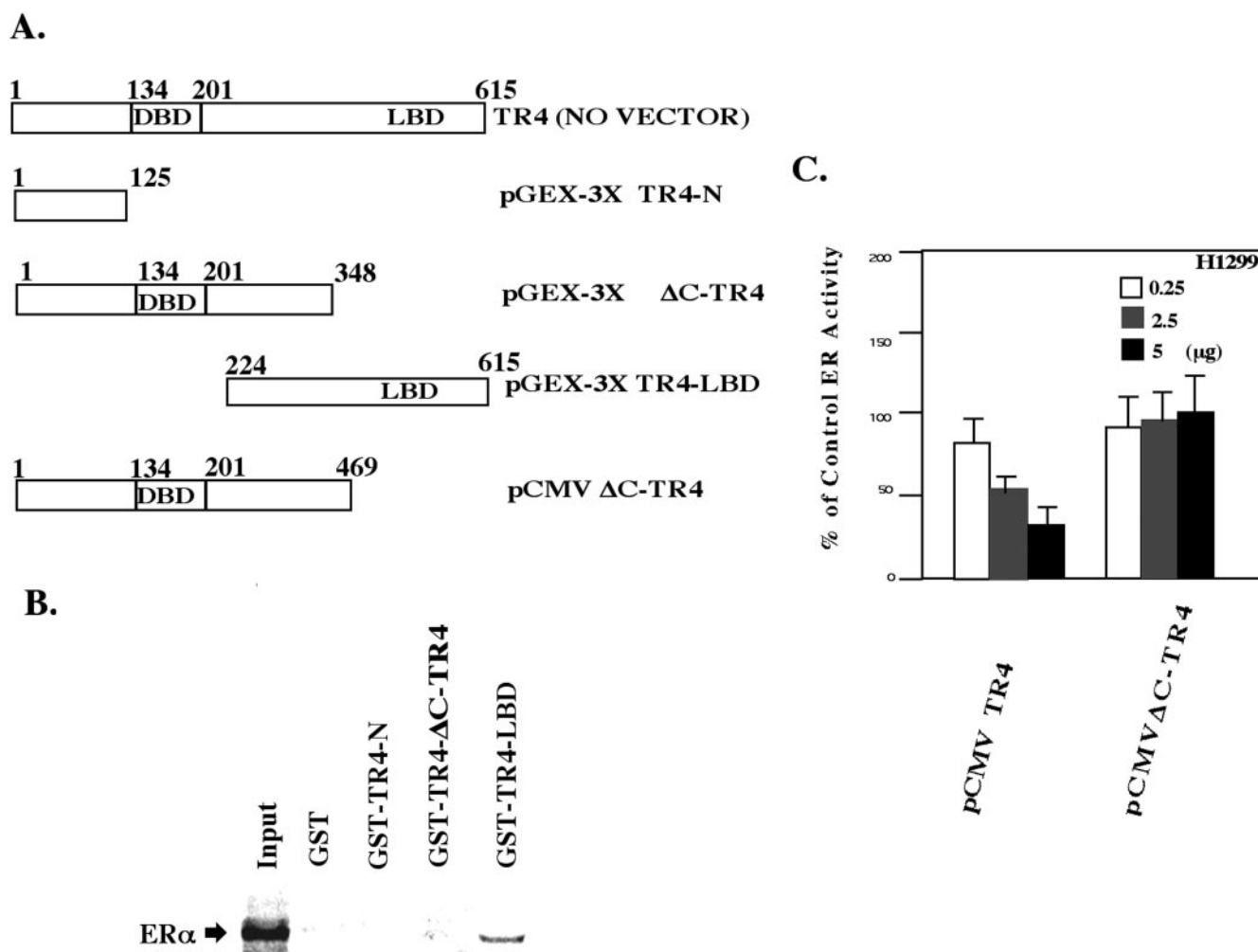


FIG. 3. TR4-LBD is essential for the suppression effect of TR4 on ER. *A*, constructs used in the GST pull-down assay and transient transfection. *B*, GST pull-down assay used ^{35}S -labeled ER and purified GST- or GST fusion protein-bound glutathione-Sepharose beads. The input represents 20% of the amount of labeled protein used in the pull-down assay. *C*, H1299 cells were cotransfected with the ERE-CAT reporter plasmid and the expression plasmid for ER α and increasing amounts of full-length TR4 or C-terminal deletion TR4 expression plasmids. Transfected cells were treated with 10 nM E2. The E2-induced CAT activity of lysates from cells transfected with ER α expression plasmid only was used as 100% control. Bars represent the means \pm S.D. of three individual experiments.

Suppression of ER Target Gene Expression in MCF-7 Cells with Tetracycline-induced TR4—So far, all TR4 suppressions of ER transactivation have been demonstrated with transient transfection of TR4 in various cell lines. To rule out potential artifacts with these transient transfection methods, we constructed a tetracycline-induced TR4 expressed MCF-7 cell line (MCF-7-TR4). As shown in Fig. 5A, addition of 2 $\mu\text{g}/\text{ml}$ Dox induces TR4 expression in MCF-7-TR4 cells. The induction of TR4 could then repress the ERE-CAT activity. In contrast, addition of 2 $\mu\text{g}/\text{ml}$ Dox in control MCF-7-pBIG cells (cells stably transfected with parent vector pBIG) showed little or no influence on the ERE-CAT (Fig. 5B). Fig. 5C further demonstrated that Dox-induced TR4 could also repress the ER endogenous target gene pS2 (22) expression, but had no influence on the β -actin gene expression, which serves as a negative control. Together, Fig. 5 clearly demonstrates that TR4 can repress ER target gene expression in the MCF-7 cells stably transfected with TR4.

TR4 Expression in MCF-7 Inhibits the Estrogen-stimulated Cell Growth and Cyclin D1 Expression—We then studied the potential physiological consequence of the TR4 suppression of ER transactivation. As it is well documented that E2/ER play pivotal roles for the breast cancer growth (23, 24), we were interested in determining whether TR4 can also modulate E2/

ER-mediated breast cancer MCF-7 cell growth. As shown in Fig. 6A, addition of 10 nM E2 stimulated cell growth in both MCF-7-TR4 and MCF-7-pBIG cells. Addition of Dox to both cells lines, however, only repressed cell growth in the MCF-7-TR4 cells, suggesting that Dox-induced TR4 could repress the E2/ER-mediated cell growth. To further dissect the potential mechanism of how TR4 repressed the E2/ER-mediated cell growth, we examined the expression of cyclin D1, a cell cycle regulator responsible for G₁-S phase transition, which has been linked to the E2/ER-mediated cell growth (25). As shown in Fig. 6B, in MCF-7-TR4 cells, addition of 10 nM E2 induced cyclin D1 expression, and this induction was repressed by adding Dox. In contrast, in MCF-7-pBIG cells there was no influence on the E2-induced cyclin D1 gene expression after adding Dox. Together, Fig. 6 suggests that TR4 may be able to repress E2/ER-mediated cell growth via modulation of the ER target genes, such as cyclin D1 expression.

DISCUSSION

Early studies in nuclear receptors suggested that TR, RAR, SHP, and chicken ovalbumin upstream promoter transcriptional factor may be able to interact with ER and modulate ER transactivation (14–16, 26). As these receptors, together with their ligands such as triiodothyronine or retinoids, may play

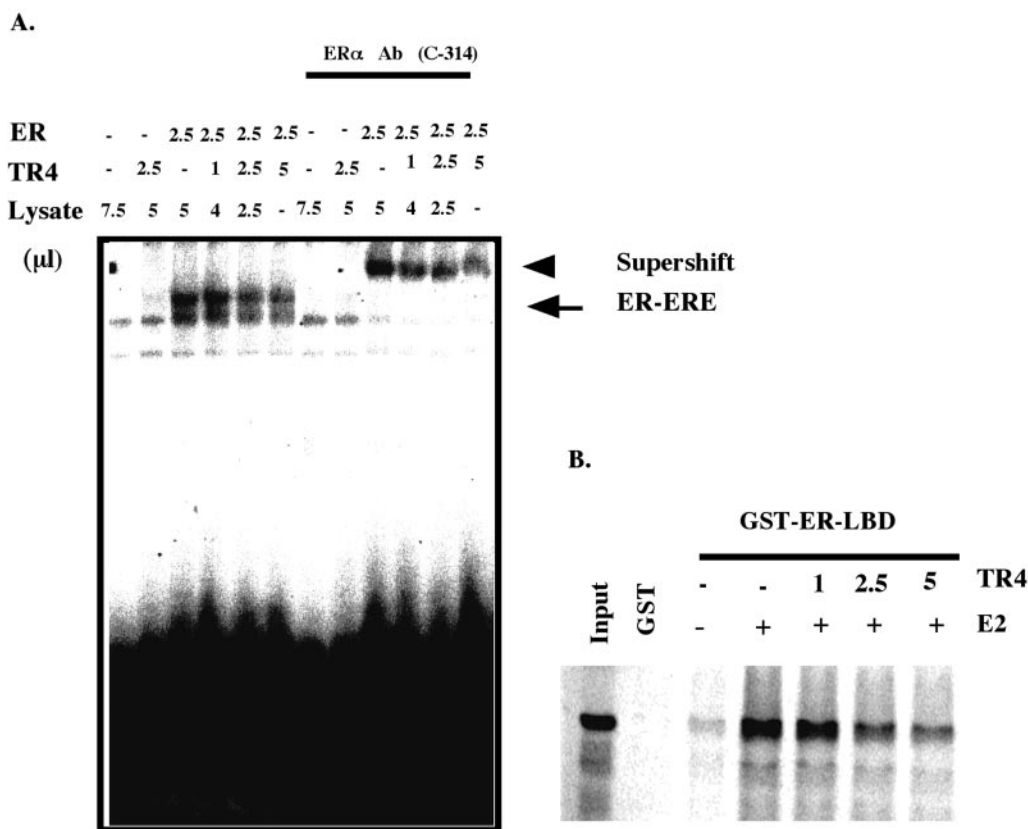


FIG. 4. *A*, 32 P-labeled ERE was either incubated with *in vitro* expressed ER with increasing amounts of *in vitro* expressed TR4 or TR4 alone as indicated at the top of each lane and resolved in 5% native gel. ER-ERE was further supershifted by ER antibody (C-314). The arrow indicates the ER-ERE complex, and the arrowhead indicates supershift complex. *B*, 35 S-labeled ER was incubated with purified GST-ER-LBD or GST-bound glutathione-Sepharose beads with increasing amounts of TR4 in the absence or presence of 1 μ M E2. The input represents 20% of the amount of labeled protein used in the pull-down assay.

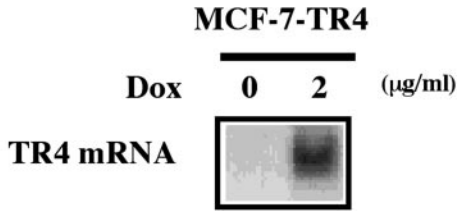
important roles in the ER-mediated cell growth (27, 28), we expect to see the pleiotropic effect of estrogen may require the cooperation of ER with a large network of nuclear receptors. The identification of TR4 as one of the ER-interacting proteins to modulate ER functions further strengthens this hypothesis. The *in vivo* effect of an individual receptor's influence on the ER function, however, may depend on the availability of whole ER interaction proteins in any single cell. For this reason, we may expect to see differential TR4 suppression effects on ER transactivation in a variety of cells, depending on the relative amount of TR4 compared with other proteins that were also able to bind and modulate ER functions.

The analysis of ER α KO mice indicated that ER α may play important *in vivo* functions, such as the growth of the adult female reproductive tract and mammary gland, the regulation of gonadotropin gene transcription, mammary neoplasia induction, and sexual behaviors. Surprisingly, ER α also plays important roles in spermatogenesis and sperm function (see review, see Ref. 29). However, the detailed mechanism for ER α to control these pathways remains unclear. Previous reports have linked TR4 function to neurogenesis (9) and spermatogenesis (30), but the real physiological roles of TR4 are still unknown. Here, we provide the evidence to support the hypothesis that the complexity of ER function could be achieved partly by the coordination with other nuclear receptors such as TR4, which further strengthens our early hypothesis that TR4 may play important physiological roles.

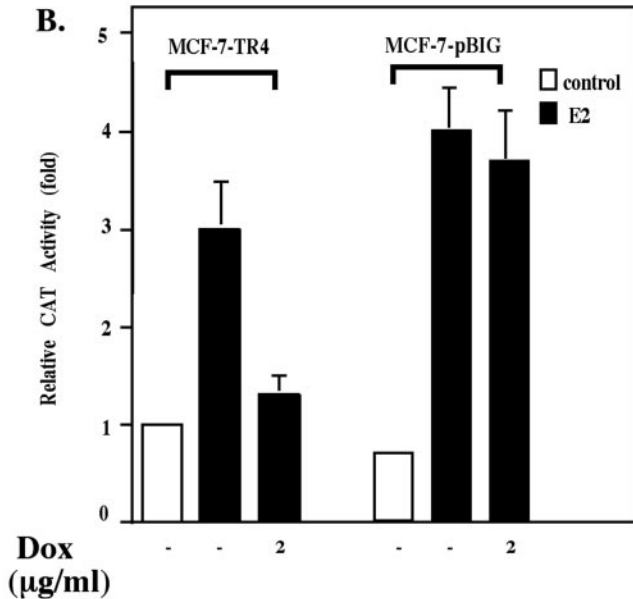
TR4 has been demonstrated to suppress many other receptors' transactivation, such as VDR, RAR, RXR, and PPAR (6, 8, 10). The suppression mechanism for these receptors' transac-

tivation has been demonstrated through the competition of TR4 with those receptors' ability to bind their hormone response elements. This competition mechanism is, therefore, different from the TR4 suppression of ER transactivation, which is through protein-protein interaction (Fig. 2). The fact that there is no extra band in the EMSA when we added TR4 alone or along with ER and incubated with 32 P-labeled ERE (Fig. 4A), clearly suggests that TR4 will not bind to 32 P-labeled ERE. Instead, the TR4 will reduce ER- 32 P-labeled ERE binding through heterodimer formation with ER, which therefore titrates out the free ER. In addition to the interruption of binding between ERE and ER, our data also demonstrated that TR4 could inhibit ER-ER homodimerization (Fig. 4B). Fig. 3 further demonstrates that TR4 may interact with ER α via its LBD. Early reports suggest that ER α is also able to form homodimers or heterodimers with other receptors through a common dimerization surface within the LBD, which is located in a conserved hydrophobic region at the N terminus of helix 10/11 (13, 31). Together, these studies suggest that both ER and TR4 can use their LBDs to interact with other receptors. Early reports also demonstrated that other receptors might bind to ER via LBD to modulate ER functions. For example, truncated estrogen receptor product-1 (TERP-1), which only contains the C-terminal region of the full-length ER, forms heterodimers with ER and inhibits ER binding to EREs (32). SHP interacts with and inhibits ER transactivation by the competition for coactivators, like RIP140 (16). In contrast to the above suppression mechanism, our data show that TR4 did not influence the binding between RIP140 and ER. Instead, TR4 modulates ER functions via interruption of ER homodimerization and ER binding to

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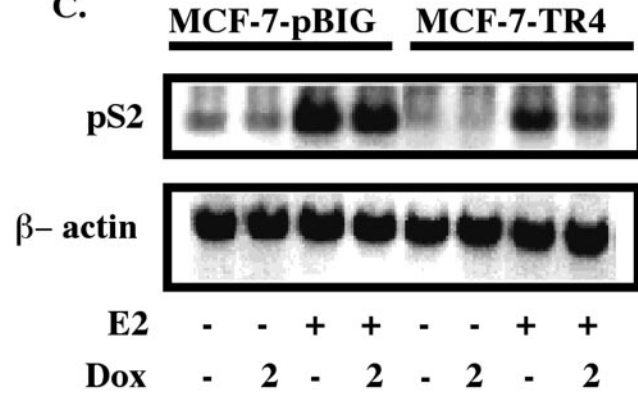


FIG. 5. Inhibition of ER endogenous target gene pS2 by Dox-induced TR4 expression. A, TR4 expression was induced by Dox in stably transfected MCF-7 cells (MCF-7-TR4). RNA from MCF-7-TR4 cells was isolated in the absence or presence of 2 µg/ml Dox. N-terminal hTR4 was used as probe to perform Northern blotting. B, MCF-7-TR4 cells and MCF-7-pBIG cells were transfected with ERE-CAT reporter plasmid. The cells were then treated with 10 nM E2 for ERE-CAT after 2 µg/ml Dox treatment for 24 h. C, RNA was isolated from MCF-7-TR4 and MCF-7-pBIG cells with or without 2 µg/ml Dox treatment for 24 h and then treated with 10 nM E2 or ethanol for 2 days. Northern blotting was performed to determine the expression level of pS2 gene. The β-actin labeling was used to demonstrate the equal loading of RNA amount.

ERs. Therefore, TR4 may represent a new category of negative coregulators of ER.

Furthermore, our TR4 stably transfected MCF-7 cells clearly demonstrate that TR4 could inhibit the expression of estrogen-induced pS2 mRNAs (22), which is specifically and directly induced by estrogens through ER at the transcriptional level in MCF-7 cells (Fig. 5C). Since the expression of the pS2 gene has been widely used as a marker to monitor the effect of estrogens, this result suggests that TR4 could suppress ER function not only occurring in cell line transient transfection experiments (Fig. 1, A and B), but also in ER target gene expression. TR4 is also able to suppress estrogen-induced cell proliferation in MCF-7 cells stably transfected with TR4 induced by Dox (Fig. 5A). This result extends our *in vitro* results and demonstrates that the consequence of the protein-protein inhibition can result in the suppression of E2/ER-induced cell growth. Lazennec *et al.* reported that estrogen-stimulated gene expression and proliferation of MCF-7 breast cancer cells could be blocked by adding a dominant-negative ER (S554-frameshifted) (33). The possible suppression mechanism, however, is different from TR4 suppression of ER functions and may involve the formation of inactive heterodimers between the dominant-negative ER and wild-type ER (34).

Estrogen-induced breast cancer cell proliferation has been linked well to the modulation of cyclin D1 that plays important roles in the cell cycle control to stimulate the G₁-S phase

progression (25, 35). Results from Fig. 6B also demonstrate that TR4 could suppress E2/ER-regulated cyclin D1 gene expression. This Dox-inducible cell model system to control expression of TR4 and the function of ER may therefore provide a nice system for studying the physiological roles of TR4 in ER target organs, such as breast and testis.

The potential impacts of this new finding could be significant, and it may allow us to modulate ER function via interrupting the binding between ER and TR4. Any compounds or small peptide(s) that mimic the interaction domain between TR4 and ER could be developed for future therapeutic uses. These compounds or peptide(s) may have minimal side effects due to unique abilities to specifically block the ER-TR4 interaction. Our preliminary data from TR4 KO mice suggest that TR4 may play an important role in the growth and reproductive systems,² and it has been well documented that ER also plays an important role in these areas. It is possible that normal growth and reproductive development and function may require the balance and coordination between these two receptors.

Tissue distribution studies indicated that ERα is expressed well in testes and epididymis (36). ERαKO mice are infertile and produce lower numbers of epididymal sperm, compared with wild-type mice at 12 weeks. Furthermore, the sperm pro-

² L. Collins and C. Chang, unpublished data.

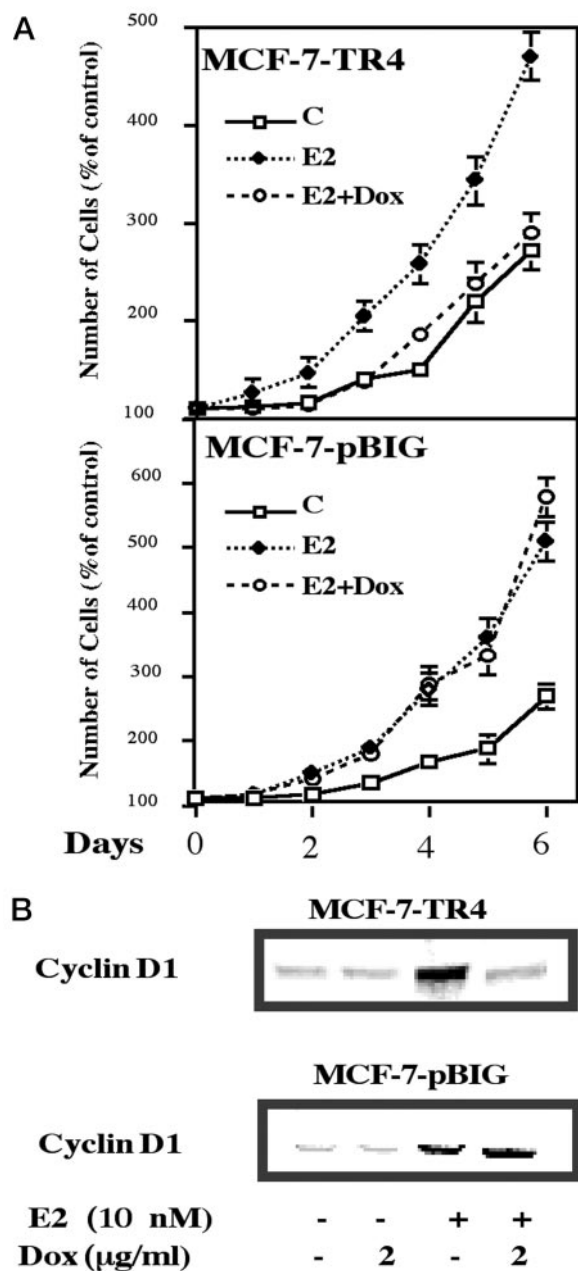


FIG. 6. Suppression of E2-induced MCF-7 cell growth and cyclin D1 expression by Dox-induced TR4 expression. A, MCF-7-TR4 and MCF-7-pBIG cells were estrogen-deprived for 4 days, then treated with 2 $\mu\text{g}/\text{ml}$ Dox treatment for 24 h, and then treated with 10 nM E2 to induce cell proliferation, and then the cells were counted at different times. The cell number after 24 h was used as 100% control. Bars represent the means \pm S.D. of three individual experiments. B, the cell lysates from MCF-7-TR4 and MCF-7-pBIG cells treated in the presence or absence of 2 $\mu\text{g}/\text{ml}$ Dox for 24 h were collected after 12 h with or without the treatment with 10 nM E2. Cyclin D1 protein was probed by cyclin D1 rabbit polyclonal Ab (H295) and detected by alkaline phosphatase-conjugated secondary Ab.

duced in the ER α KO mice have obvious defects and are unable to fertilize wild-type oocytes (37). These studies indicated that ER α plays important roles in the testes function and spermatogenesis. Interestingly, our early studies of tissue distribution studies not only showed that TR4 is highly expressed in testes, but that it is also strongly linked to the defective spermatogenesis found in rhesus monkey in either surgery-induced cryptorchid testis (38) or testis injected with high dose testoster-

one.³ In addition, we recently also found that the sperm number and motility were decreased in our TR4KO male mice, which are similar to ER α KO mice.² Whether suppression of ER by TR4 plays any major roles in the spermatogenesis and other testis functions, therefore, remains an interesting topic for future study.

In conclusion, the discovery that the E2/ER signaling pathway can be interrupted by TR4 through protein-protein interaction represents a unique TR4 function. Future studies may further expand the roles of TR4 on the E2/ER function in the reproductive system.

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